THERMOSTABLE L-ARABINOSE ISOMERASE AND PROCESS FOR PREPARING D-TAGATOSE

Related Applications

[0001] This application is a continuing application under 35 U.S.C. § 365(c) claiming the benefit of the filing date of PCT Application No. PCT/KR01/02243 designating the United States, filed December 22, 2001. The PCT Application was published in English as WO 02/052021 A1 on July 4, 2002, and claims the benefit of the earlier filing date of Korean Patent Application Nos. 2000/80608, filed December 22, 2000, and 2000/80711, filed December 18, 2001. The contents of Korean Patent Application Nos. 2000/80608 and 2000/80711, and the international application No. PCT/KR01/02243 and the publication 02/052021 A1 are incorporated herein by reference in their entirety.

Background of the Invention

Field of the Invention

[0002] The present invention relates generally to production of an enzyme for use in production of a sweetener. More particularly, the present invention relates to production of arabinose isomerase and tagatose.

Description of the Related Art

[0003] In recent years, growing concerns about health have led much research effort to the development of healthful foods. As one of the above efforts, sugar alcohols have been proposed as sweeteners which can substitute sugar, known to cause adult diseases, and are practically being used. Since the said sweeteners are known to have adverse side effects such as causing diarrhea when ingested more than certain amount, there is an urgent need to develop substitutional sweeteners without harmful effects.

[0004] Among substitutional sweeteners which have little side effect, tagatose, a keto-sugar of galactose, has similar sweetness to D-fructose, and has known not to be absorbed or metabolized in the body, making tagatose a safe low-caloric substitutional sweetener for sugar. Also, it has been reported that tagatose can be employed as an

intermediate for the preparation of useful optically active isomers, detergents and cosmetics, also, as an additive or raw material for the synthesis of drugs, especially, its ability to lower blood sugar level renders tagatose a therapeutic and preventive agent for diabetes, and a low caloric diet agent.

[0005] Currently, tagatose is mostly prepared via chemical synthesis from galactose (see: USP 5,002,612), which comprises the steps of isomerization of galactose catalyzed by metal hydroxide in the presence of inorganic salts to form an intermediate of metal hydroxide-tagatose complex, and neutralization of the complex by adding acid to yield final product, tagatose.

[0006] Alternative method for manufacturing tagatose is an enzymatic method in which galactose is converted into tagatose via conversion of aldose or aldose derivatives into ketose or ketose derivatives. Especially, it has been reported that arabinose isomerase which catalyzes the conversion reaction of L-arabinose into L-ribulose can be employed for production of tagatose in vitro using galactose as a substrate. However, the yield of tagatose produced by arabinose isomerase from galactose is as low as 20%, hindering industrial application of conversion process of galactose into tagarose. Although the method for manufacturing tagatose from milk or cheese has been developed (see: USP 6,057,135), again, low yield is the limitation for its industrial use.

[0007] Under the circumstances, there are strong reasons for exploring and developing a novel enzyme which can produce tagatose with high yield.

Summary of the Invention

[0008] An aspect of the present invention provides an isolated polynucleotide coding for an arabinose isomerase from *Thermatoga neapolitana*. The isolated polynucleotide has the sequence of SEQ. ID NO: 3.

[0009] Another aspect of the present invention provides an expression vector, which comprises the above-described isolated polynucleotide. The expression vector is pTNAI.

[0010] Another aspect of the present invention provides a host cell transformed with the above-described expression vector. The host cell is *E. coli*. The host cell is E. coli BL21/DE3 (pTNAI) deposited as Accession No. KCCM-10231.

[0011] Another aspect of the present invention provides an isolated polypeptide of arabinose isomerase isolated from *Thermatoga neapolitana*.

[0012] Still another aspect of the present invention provides an isolated polypeptide of arabinose isomerase encoded by the above-described polynucleotide. The arabinose isomerase has the amino acid sequence of SEQ. ID NO: 4. The isolated polypeptide further comprises a solid support. The solid support is a silica bead.

[0013] Still another aspect of the present invention provides a method of producing an arabinose isomerase. The method comprises: providing the above-described host cell; and culturing the host cell in a medium, thereby producing an arabinose isomerase. The method further comprises purifying or isolating the arabinose isomerase. The host cell is *E. coli* BL21/DE3 (pTNAI) deposited as Accession No. KCCM-10231.

[0014] Still another aspect of the present invention is an arabinose isomerase produced by the above-described method.

[0015] A still further aspect of the present invention provides a method of producing tagatose. The method comprises: providing the above-described isolated polypeptide; and admixing the arabinose isomerase with galactose, thereby causing a reaction and producing tagatose. The reaction is carried out at a pH from about 5 to about 8. The reaction is carried out at a temperature from about 50°C to about 100°C. The reaction is carried out at a temperature from about 70°C to about 95°C. The method of Claim 17, wherein the isolated polypeptide is attached to a solid support. The solid support is a silica bead. The reaction is carried out at a temperature of about 80°C.

Brief Description of the Drawings

[0016] The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings.

[0017] Figure 1 is a schematic diagram showing the construction strategy of an expression vector containing arabinose isomerase gene of the invention.

[0018] Figure 2 is a graph showing activity profile of arabinose isomerase of the invention depending on temperature.

[0019] Figure 3 is a graph showing thermostability of arabinose isomerase of the invention.

[0020] Figure 4 is a graph showing the time course of conversion rate of galactose into tagatose by arabinose isomerase of the invention at various reaction temperatures.

[0021] Figure 5 is a graph showing the time course of changes in thermostability of immobilized arabinose isomerase of the invention.

Detailed Description of Embodiments

[0022] The present inventors have made an effort to develop an enzyme which can produce tagatose with high yield, and have found that tagatose can be produced with high yield from galactose by employing a recombinant arabinose isomerase produced from *E. coli* transformed with recombinant vector containing arabinose isomerase gene derived from *Thermotoga neapolitana* 5068.

[0023] To prepare thermophilic or thermostable arabinose isomerase for industrial use, the present inventors have cloned a gene coding for arabinose isomerase from genomic DNA of *Thermotoga neapolitana* 5068 (DSM 5608) and analyzed nucleotide sequence and deduced amino acid sequence from the said gene. The nucleotide sequence and deduced amino acid sequence of the gene encoding arabinose isomerase of an embodiment of the present invention (SEQ ID NO: 3) has shown to have 83.2% and 94.8% homology, respectively, to those of the putative arabinose isomerase gene of *Thermotoga maritima* of which entire nucleotide sequence has been verified via genome project.

[0024] For high level expression of the said cloned arabinose isomerase in *E. coli*, the gene coding for the enzyme was inserted into an expression vector pET22b(+) (Novagen, U.S.A.) to construct a recombinant expression vector pTNAI, which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3 (pTNAI)" and deposited with an international depository authority, the Korean Culture Center of Microorganisms (KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on December 4, 2000 as accession no. KCCM-10231.

[0025] The said *E. coli* BL21/DE3 (pTNAI) was grown to obtain recombinant arabinose isomerase, which was characterized to have optimum pH of 7.0, optimum reaction

temperature of 85°C. Furthermore, over 80% of remaining activity was measured after 2 hour heat treatment at 80°C, indicating that the enzyme is exceedingly heat stable.

[0026] Tagatose can be produced by employing arabinose isomerase of the embodiment of the present invention prepared from *E. coli* transformed with a recombinant expression vector containing the gene for arabinose isomerase derived from *Thermotoga* sp., and galactose as a substrate, under a condition of pH 5 to 8, more preferably pH 6 to 8, most preferably pH 7, and 60 to 100°C, more preferably 70 to 95°C, most preferably 85°C.

[0027] Aqueous solution of galactose was subjected to isomerization reaction employing recombinant arabinose isomerase of the embodiment of the present invention, and it has been found that conversion rate into tagatose was over 68% at 80°C.

[0028] When the said recombinant arabinose isomerase is employed for industrial production of tagatose, soluble form of the enzyme may be employed, nevertheless, it is more preferable to immobilize the enzyme on the beads used in industry. For example, in case of the recombinant arabinose isomerase of the embodiment of the present invention immobilized on silica beads, the remaining activity was measured to be over 80% of original activity after 20 day-heat treatment at 90°C, thus, it can be applied for thermal process over 80°C in industry.

Examples

[0029] Embodiments of the present invention are further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Cloning of arabinose isomerase gene

[0030] *Thermotoga neapolitana* 5068 (DSM 5068) was grown under an anaerobic condition and cells were harvested by centrifugation at 8000xg for 10 minutes. Genomic DNA isolated from the cells harvested above was partial digested with Sau3AI (TaKaRa Biotechnology, Japan) to obtain 12kb or shorter fragments of DNA. The DNA fragments were inserted into ZAP Expression Vector (Stratagene, U.S.A.) and packaged to prepare a genomic library of *Thermotoga neapolitana* 5068. Nucleotide sequences of the genes for conventional thermophilic or thermostable arabinose isomerase were analyzed to prepare

primer araAF: 5'-ATGATCGATCTCAAACAGTATGAG-3' (SEQ ID NO: 1) and primer araAR: 5'-TCATCTTTTTAAAAGTCCCC-3' (SEQ ID NO: 2), which were used in PCR for the preparation of probes for DNA-DNA hybridization. The genomic library prepared above was screened for DNA fragments containing arabinose isomerase gene by DNA-DNA hybridization to obtain a recombinant vector containing a gene encoding arabinose isomerase of *Thermotoga neapolitana* 5068. The nucleotide sequence of arabinose isomerase gene (SEQ ID No: 3) cloned above and the deduced amino acid sequence (SEQ ID No: 4) from the said gene were compared with those of known arabinose isomerase genes, respectively (see: Table 1).

Table 1: Comparison of homology between arabinose isomerase of one embodiment of the present invention and known arabinose isomerases

Strain	Gene Sequence (homology, %)	Amino Acid Sequence (homology, %)
Thermotoga maritima	83.2	94.8
Bacillus stearothermophilus	61.9	62.8
Bacillus halodurans	59.1	59.0
Bacillus subtilis	58.6	55.5
Salmonella typhimurium	57.8	54.5
Escherichia coli	59.0	54.3
Mycobacterium smegmatis	56.3	50.7

[0031] As shown in Table 1, it has been found that the arabinose isomerase of the embodiment of the present invention is a novel enzyme which has 83.2% homology of nucleotide sequence and 94.8% homology of amino acid sequence to the sequences of published putative arabinose isomerase of *Thermotoga maritima*, respectvely.

Example 2: Preparation of recombinant expression vector and recombinant *E. coli*

[0032] In order to obtain high level expression of the said thermostable arabinose isomerase in *E. coli* using the arabinose isomerase gene obtained in Example 1, the said gene

was inserted into an expression vector pET 22b(+) (Novagen, U.S.A.) double-digested with *NdeI* and *EcoRI* to construct a recombinant expression vector pTNAI (see: Figure 1), which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3 (pTNAI)" and deposited with an international depository authority, the Korean Culture Center of Microorganisms (KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on December 4, 2000 as accession no. KCCM-10231.

Example 3: Expression of recombinant arabinose isomerase

[0033] The recombinant *E. coli* BL21/DE3 (pTNAI) (KCCM-10231) prepared in Example 2 was inoculated into LB (Luria-Bertani) medium (1% v/v) and incubated at 37°C for 2 hours, to which lactose was added to a final concentration of 1mM and expression of recombinant arabinose isomerase was induced for 12 hours. For assay of expressed arabinose isomerase, cells were collected by centrifugation at 8000xg for 10 minutes, resuspended in 10ml of 100mM MOPS buffer (4-morpholinepropanesulfonic acid, pH 7.0), and then disrupted by sonication to obtain crude enzyme, with which galactose isomerization reaction was carried out. Galactose isomerization was performed by mixing 100μl of the said crude enzyme solution with 40mM (final concentration) galactose as a substrate, followed by adding 1ml of enzyme reaction buffer (100mM MOPS buffer, pH 7.0) containing cofactors (1mM $MnCl_2$, 1mM $CoCl_2$) and incubating at 85°C for 20 minutes. The product of the above reaction was detected using cysteine-carbazole-sulfuric acid method (see: Dische, Z., and E. Borenfreund., A New Spectrophotometric Method for the Detection and Determination of Keto Sugars and Trioses, *J. Biol. Chem.*, 192:583-587, 1951), and it has been found that normal galactose isomerization has been undergone.

Example 4: Purification of recombinant arabinose isomerase

[0034] For purification of recombinant arabinose isomerase expressed by the method described in Example 3, cells were collected by centrifugation at 8000xg for 19 minutes and cell wall of *E. coli* was disrupted by sonication, which was followed by centrifugation at 20,000xg for 20 minutes to obtain supernatant. Then, the said supernatant was heat-treated at 85°C for 20 minutes, centrifuged at 20,000xg for 20 minutes to get rid of

precipitate, and the supernatant was further purified by ammonium sulfate-mediated precipitation and finally ion-exchange column chromatography (Q-Sepharose Fast Flow, Pharmacia, Sweden). pH dependancy of the said purified enzyme was analyzed and optimum pH was found to be around 7.0.

Example 5: Optimum pH and optimum temperature of recombinant arabinose isomerase

[0035] Activity of the purified recombinant arabinose isomerase prepared in Example 4 was analyzed on galactose substrate and optimum pH was found to be around 7.0. Optimum temperature for isomerization reaction was determined using the same method as described in Example 3. The tested reaction temperatures for galactose isomerization were 60, 70, 75, 80, 85, 90 and 100°C, and maximum activity was obtained around 85°C (see: Figure 2).

Example 6: Thermostability of recombinant arabinose isomerase

[0036] To assess the thermostability of recombinant arabinose isomerase of the embodiment of the present invention, crude enzyme prepared in Example 3 was heat-treated at 60, 70, 80 and 90°C for 10, 20, 30, 60, 90 and 120 minutes respectively, and remaining activity of recombinant arabinose isomerase for isomerization was determined as described in Example 3 (see: Figure 3). As shown in Figure 3, it has been found that over 80% of enzyme activity was remained after 2 hour heat-treatment at 80°C.

Example 7: Conversion rate of galactose into tagatose at various temperature

[0037] By employing recombinant arabinose isomerase of the embodiment of the present invention, the conversion rate of galactose into tagatose was determined at various temperatures and various time points. Substrate used was 10mM galactose instead of 40mM galactose in enzyme reaction mixture in Example 3. After incubation at 60, 70, 80 and 90°C for 20 hours, tagatose yield was determined employing BioLC (see: Table 2 and Figure 4).

Table 2: Conversion rate of galactose into tagatose at various temperature

Enzyme Reaction Temperature	60°C	70°C	80°C	90°C
Conversion Rate into Tagatose	31.7	40.4	68.1	57.4

[0038] As shown in Table 2 and Figure 4, the higher the reaction temperature was, the higher tagatose yield was obtained, and conversion rate into tagatose was as high as 68% at 80°C.

Example 8: Immobilization of arabinose isomerase and improvement of thermostability

[0039] Arabinose isomerase was immobilized on silica beads, heat-treated under an aqueous condition at 90°C and the remaining activity was determined at various time points (see: Figure 5). As shown in Figure 5, remaining activity of the immobilized enzyme was over 80% after 20 day-heat treatment at 90°C and over 60% after 30 day-heat treatment, indicating that the immobilized arabinose isomerase of the embodiment of the present invention can be applied for thermal process in industry.

[0040] As clearly illustrated and demonstrated above, the present invention provides, among other things, a novel gene coding for L-arabinose isomease derived from *Thermotoga neapolitana* 5068, a thermostable arabinose isomerase expressed from the said gene, a recombinant expression vector containing the said gene, a microorganism transformed with the said expression vector, a process for preparing thermostable arabinose isomerase from the said transformant and a process for preparing D-tagatose employing the said enzyme. Since the recombinant arabinose isomerase of the embodiment of the present invention is highly thermostable and can produce tagatose with high yield at high temperature, it can be efficiently applied in pharmaceutical and food industries.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL
(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on
Name of depositary institution Korean Culture Center of Microorganisms (KCCM)
Address of depositary institution (including postal code and country) Korean Culture Center of Microorganisms (KCCM) 361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu Seoul, 120-091, Republic of Korea
Date of deposit Dec. 04, 2000 | Accession Number KCCM-10231
C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information continues on an additional sheet ☐
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*
E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*
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